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1 **Original Article**

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4 **Lipopolysaccharide and toll-like receptor 4 in dogs with congenital**
5 **portosystemic shunts**

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30

31 **Abstract**

32 Surgical attenuation of a congenital portosystemic shunts (CPSS) results in
33 increased portal vein perfusion, liver growth and clinical improvement. Portal
34 lipopolysaccharide (LPS) is implicated in liver regeneration via toll-like receptor
35 (TLR) 4 mediated cytokine activation. The aim of this study was to investigate factors
36 associated with LPS in dogs with CPSS.

37 Plasma LPS concentrations were measured in the peripheral and portal blood
38 using a limulus amoebocyte lysate (LAL) assay. LPS concentration was significantly
39 greater in the portal blood compared to peripheral blood in dogs with CPSS ($P =$
40 0.046) and control dogs ($P = 0.002$). LPS concentrations in the peripheral ($P = 0.012$)
41 and portal ($P = 0.005$) blood of dogs with CPSS were significantly greater than those
42 for control dogs. The relative mRNA expression of cytokines and TLRs was
43 measured in liver biopsies from dogs with CPSS using quantitative PCR. TLR4
44 expression significantly increased following partial CPSS attenuation ($P = 0.020$).
45 TLR4 expression was significantly greater in dogs that tolerated complete CPSS
46 attenuation ($P = 0.011$) and those with good portal blood flow on pre-attenuation ($P =$
47 0.004) and post-attenuation ($P = 0.015$) portovenography. Serum IL-6 concentration
48 was measured using a canine specific ELISA and significantly increased 24 h
49 following CPSS attenuation ($P < 0.001$).

50 Portal LPS was increased in dogs with CPSS, consistent with decreased
51 hepatic clearance. TLR4 mRNA expression was significantly associated with portal
52 blood flow and increased following surgery. These findings support the concept that
53 portal LPS delivery is important in the hepatic response to surgical attenuation. Serum
54 IL-6 significantly increases following surgery, consistent with LPS stimulation via
55 TLR4, although this increase might be non-specific.

56 *Keywords:* Lipopolysaccharide; Toll like receptor 4; IL-6; Liver; Dog; congenital
57 portosystemic shunts
58

59 **Introduction**

60 A congenital portosystemic shunt (CPSS) is an abnormal vessel connecting
61 the portal venous system to the systemic venous system (Payne et al., 1990). A CPSS
62 allows blood from the splanchnic viscera to bypass the liver, resulting in portal vein
63 hypoperfusion and hence liver hypoplasia and hepatic insufficiency. Surgical CPSS
64 attenuation is recommended to restore normal portal blood flow. Successful CPSS
65 attenuation results in resolution of clinical signs, improvements in hepatic function
66 and portal perfusion and increased liver volume (Hunt and Hughes, 1999; Kummeling
67 et al., 2010; Lee et al., 2006; Stieger et al., 2007). These findings suggest that the
68 return of normal hepatic size and function is achieved by liver regeneration. We have
69 previously shown that markers of hepatocyte replication are associated with the
70 degree of liver development and the response to surgery in dogs with CPSS,
71 supporting a role for hepatic regeneration (Tivers et al., 2014a).

72

73 Hepatic portal blood flow contributes 80% of the afferent liver blood flow and
74 is vital for normal liver regeneration in people, rodents and dogs (Mathie et al., 1979;
75 Michalopoulos, 2007). In experimental partial hepatectomy (PH) in pigs and rats,
76 removal of two thirds of the liver mass caused an effective increase in hepatic portal
77 blood flow (Kahn et al., 1984; Rice et al., 1977). It is unclear whether liver
78 regeneration is stimulated by the increase in blood flow or by increased delivery of
79 hepatotrophic substances in the portal blood (Mortensen and Revhaug, 2011). This
80 increase in portal blood flow relative to liver mass is similar to that observed
81 following CPSS attenuation. Intuitively, the response to CPSS attenuation is likely to
82 be governed by similar factors.

83

84 Lipopolysaccharide (LPS) or endotoxin is a component of the cell wall of
85 Gram-negative bacteria and is released following bacterial death. Gram-negative
86 bacteria are present in the small intestine and therefore LPS is absorbed from the gut
87 and into the portal vein (Peterson et al., 1991; Howe et al., 1997). LPS has been
88 shown to play a positive role in liver regeneration in rodent models (Cornell, 1985a,
89 b, 1990; Gao et al., 1999). Kupffer cells are specialised hepatic macrophages that bind
90 LPS entering the liver via the portal vein (Freudenberg et al., 1982). LPS acts on
91 Kupffer cells by binding to toll-like receptor (TLR) 4 (Fenton and Golenbock, 1998).
92 Kupffer cells produce IL-6 and tumour necrosis factor (TNF) α after stimulation with
93 LPS in rodents and these cytokines are implicated in the early stages of liver
94 regeneration (Carswell et al., 1975; Shirahama et al., 1988; Decker et al., 1989; Hori
95 et al., 1989; Busam et al., 1990). Activation of the cytokine network via LPS
96 stimulation of Kupffer cells has been suggested as the stimulus for liver regeneration
97 (Fausto, 2006a). Therefore, it is possible that LPS contributes to triggering the hepatic
98 response to CPSS attenuation.

99

100 The aim of this study was to investigate whether factors involved in LPS
101 metabolism are increased after surgical attenuation of canine CPSSs. The first aim
102 was to measure the concentration of LPS in portal blood compared with peripheral
103 blood in dogs with CPSS at the time of surgery and control dogs. The second aim was
104 to measure the hepatic mRNA expression of inflammatory cytokines and TLRs in
105 dogs with CPSS, before and after partial attenuation. The third aim was to measure
106 the serum IL-6 concentration before and immediately after CPSS attenuation. The
107 hypotheses tested were that plasma LPS concentration would be significantly greater
108 in the portal blood compared with the peripheral blood and that gene expression and

109 IL-6 concentration would significantly change in response to surgical CPSS
110 attenuation.

111

112 **Materials and methods**

113 *Clinical management*

114 Dogs with CPSS were prospectively recruited between August 2007 and
115 October 2011. The Ethics Committee of the Royal Veterinary College granted ethical
116 approval (original approval on 4th June 2004 and updated 22nd October 2010, URN
117 2010 1058) and owners gave full, informed consent. Dogs were treated surgically via
118 suture attenuation of their CPSS as previously described (Lee et al., 2006). Dogs that
119 did not tolerate complete attenuation, due to intra-operative portal hypertension, had
120 partial suture attenuation. Dogs treated with partial attenuation had repeat surgery
121 approximately 3 months post-operatively.

122

123 Portovenography was performed before and after temporary complete CPSS
124 attenuation at each surgery to assess the development of the intrahepatic portal
125 vasculature as previously described (Lee et al., 2006). Grade was determined
126 according to the number of generations of intrahepatic portal vessels that were visible
127 on a scale of 1 - 4 (Lee et al., 2006). Portovenogram grades of 1 and 2 represented
128 poor portal blood flow and portovenogram grades of 3 and 4 represented good portal
129 blood flow (Tivers et al., 2014b).

130

131 Healthy experimental Beagle dogs that had been humanely destroyed for
132 reasons unrelated to hepatic disease were used as controls for all parts of the study.

133 Dogs undergoing exploratory laparotomy for reasons unrelated to CPSS were also
134 included as controls for the measurement of serum IL-6 only.

135

136 *Plasma LPS concentration*

137 Paired residual blood samples were taken peri-operatively from the jugular
138 vein and mesenteric vein of dogs with CPSS. Residual samples were available as a
139 consequence of placing a jugular central venous catheter pre-operatively and a
140 mesenteric catheter intra-operatively for the measurement of portal pressures and for
141 mesenteric portovenography. Blood samples were taken from Beagle control dogs
142 from the jugular vein immediately before and from the portal vein immediately
143 following euthanasia. Samples were collected into heparinised, glass, pyrogen free
144 tubes (Associates of Cape Cod) and the plasma was separated and stored at -80 °C.

145

146 A limulus amebocyte lysate (LAL) assay using pyrochrome chromogenic
147 reagent, reconstituted with glucashield beta glucan inhibiting buffer (Associates of
148 Cape Cod) was used to measure the plasma LPS concentration. Samples were heated
149 at 75 °C for 10 min and diluted 1:200 with LAL reagent water (Associates of Cape
150 Cod). Samples were analysed in triplicate using an ELx808 absorbance microplate
151 reader (BioTek). Sample concentration was calculated from the standard curve using
152 Gen5 V1.07.5 software (BioTek).

153

154 *qPCR Gene Expression*

155 For dogs with CPSS, at each surgery a liver biopsy was taken for routine
156 diagnostic purposes and a portion was placed in RNAlater (Sigma-Aldrich) and stored
157 according to manufacturer's instructions. Liver tissue was taken from Beagle control

158 dogs immediately following euthanasia and stored in the same way. Routine
159 histopathology was performed on sections stained with haematoxylin and eosin.

160

161 RNA was extracted from approximately 20-30 mg of each hepatic sample
162 using the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich). The tissue
163 was homogenised in 500 μ L Lysis Solution using a Mixer Mill MM 300 (Retsch). An
164 in-solution DNase digestion was performed using the Ambion TURBO DNA-free Kit
165 (Life Technologies) to remove any contaminating DNA. RNA quality and quantity
166 was assessed by microfluidic capillary electrophoresis using the Agilent 2100
167 Bioanalyser (Agilent Technologies). The median RNA integrity number was 8.3
168 (range, 7.1-9.2). No samples had genomic DNA contamination. Two separate cDNA
169 were synthesised from each RNA sample using a mixture of random hexamer and
170 oligo (dT)₁₅ primers (Promega) and IMProm-II reverse transcriptase enzyme
171 (Promega). Where possible, the amount of RNA template for cDNA synthesis was
172 standardised at 1 μ g. The cDNA was diluted to a final volume of 100 μ L with
173 nuclease-free water and stored at -20 °C before further use.

174

175 Relative hepatic expression of five genes related to hepatic LPS signalling (IL-
176 1 β , IL-6, TNF α , TLR2 and TLR4) was measured using quantitative polymerase chain
177 reaction (qPCR). Published canine specific primers for the genes of interest (Wang et
178 al., 2007; House et al., 2008) and four liver specific reference genes, hydroxymethyl-
179 bilane synthase, ribosomal protein L13a, ribosomal protein L32 and ribosomal protein
180 S18, were used (Peters et al., 2007; Table 1).

181

182 For quantification, each liver sample had two cDNA samples analysed in
183 duplicate. Reactions were carried out in 25 μ L volumes using a Bio-Rad CFX96 Real-
184 Time PCR Detection System thermocycler (Bio-Rad Laboratories). Each reaction
185 consisted of 1 μ L cDNA as the template with Immobuffer (1 \times concentration), Hi-
186 Spec Additive (1 \times concentration), dNTP (final concentration 1 mM), magnesium
187 chloride (final concentration 2.5mM for genes of interest, 4.5mM for reference
188 genes), 1 unit Immolase DNA polymerase (Bioline) and EvaGreen dye (Biotium; 0.06
189 \times diluted 1:4 with nuclease-free water). Samples were incubated at 95 $^{\circ}$ C for 10 min
190 followed by 40 cycles of denaturation at 94 $^{\circ}$ C for 30 s, annealing at 55 $^{\circ}$ C for 30 s
191 and elongation at 72 $^{\circ}$ C for 10 s. An appropriate primer-dimer melting temperature
192 for 1 s was programmed before fluorescence readings were taken at the end of each
193 cycle. A melting curve analysis from 65 $^{\circ}$ C to 95 $^{\circ}$ C with a plate read every 0.5 $^{\circ}$ C
194 was performed at the end of 40 cycles. Bio-Rad CFX Manager Software (Bio-Rad)
195 was used for initial qPCR analysis.

196

197 Analysis of raw real-time data was performed using GenEx professional
198 version 4.4.2 software (Multid Analyses). Relative gene expression was quantified as
199 previously described (Vandesompele et al., 2002). Quantification cycle (Cq) values
200 were corrected using the calculated efficiencies for each primer set. Normalisation of
201 each sample Cq for the genes of interest was performed relative to the geometric
202 normalisation of the four reference genes. The relative expression of the mRNA of
203 each gene of interest in each cDNA sample was then calculated using the normalised
204 Cq of each sample relative to the average Cq of all of the samples. For each gene the
205 following comparisons were made: CPSS compared to control; partial attenuation

206 compared to complete attenuation; before and after partial attenuation (paired
207 samples).

208

209 *Serum IL-6 concentration*

210 Blood samples were taken from both dogs with CPSS and control dogs that
211 underwent exploratory laparotomy pre-operatively for diagnostic purposes and after
212 surgery in dogs with CPSS for post-operative monitoring. Where available, residual
213 blood was used for the study. Residual blood samples were also taken immediately
214 before euthanasia in Beagle control dogs. The serum was separated and stored at -80
215 °C.

216

217 A Quantikine Canine ELISA Kit (R and D Systems) was used to measure the
218 serum concentration of IL-6 (Song et al., 2012). Samples were analysed in duplicate
219 using an ELx808 absorbance microplate reader (BioTek). Sample concentration was
220 calculated from the standard curve using Gen5 V1.07.5 software (BioTek).

221

222 *Statistical analysis*

223 Analysis was performed using PASW Statistics 18.0.0 statistical software
224 package (Education SPSS, IBM). Continuous data were visually assessed for
225 normality. Median and range were reported for skewed data, which was compared
226 with the Mann Whitney U test or the Wilcoxon signed-ranks test as appropriate.
227 Repeated measures were compared with the Friedman's two-way analysis of variance
228 by ranks with pair-wise comparison. The LPS concentration and qPCR data was
229 transformed to normal distribution (square root or logarithm). The data was then

230 compared with an independent *t* test or paired sample *t* test. Significance was set at
231 the 5% level ($P \leq 0.05$).

232

233 **Results**

234 *Plasma LPS concentration*

235 Paired peripheral and portal plasma samples from 13 dogs with CPSS were
236 included. The following breeds were included: Bichon Frise ($n=2$), Labrador ($n=2$),
237 Border terrier ($n=1$), Cavalier King Charles spaniel ($n=1$), Crossbreed ($n=1$), Dogue
238 de Bordeaux ($n=1$), German shepherd dog ($n=1$), Miniature Schnauzer ($n=1$),
239 Springer spaniel ($n=1$), West Highland white terrier ($n=1$), Yorkshire terrier ($n=1$).
240 The median age was 295 days (range, 125-1835 days). Nine dogs (69.2%) had an
241 extrahepatic CPSS and four (30.8%) had an intrahepatic CPSS.

242

243 Paired peripheral and portal plasma samples from nine healthy Beagles were
244 included as control dogs. The median age was 1136 days (range, 497-1660 days),
245 which was significantly greater than dogs with CPSS ($P = 0.036$).

246

247 For dogs with CPSS, the median LPS concentration in the portal blood was
248 0.453 endotoxin units (EU)/mL (range, 0.117-1.418 EU/mL), which was significantly
249 greater than that in the peripheral blood (0.239 EU/mL; range, 0.056-1.410 EU/mL; P
250 = 0.046; Fig. 1). For Beagle control dogs, the median LPS concentration in the portal
251 blood was 0.184 EU/mL (range, 0.126-0.565 EU/mL), which was significantly greater
252 than that in the peripheral blood (0.131 EU/mL; range, 0.061-0.187 EU/mL; $P =$
253 0.002; Fig. 1). The LPS concentrations in the peripheral blood ($P = 0.012$) and portal

254 blood ($P = 0.005$) of dogs with CPSS were both significantly greater than for Beagle
255 control dogs (Fig. 1).

256

257

258 *qPCR gene expression*

259 Liver samples obtained at the first surgery were available from 49 dogs. The
260 following breeds were included: Yorkshire terrier ($n=7$), Crossbreed ($n=6$), Labrador
261 ($n=5$), Miniature Schnauzer ($n=5$), West Highland white terrier ($n=5$), Cocker spaniel
262 ($n=4$), Jack Russell terrier ($n=3$), Bichon Frise ($n=2$), Golden retriever ($n=2$), Lhasa
263 Apso ($n=2$), Pug ($n=2$), Chihuahua ($n=1$), Hovawart ($n=1$), Irish Setter ($n=1$), Norfolk
264 terrier ($n=1$), Old English sheepdog ($n=1$), Staffordshire bull terrier ($n=1$). The
265 median age was 275 days (range, 97-4374 days). Thirty-eight (77.6%) dogs had an
266 extrahepatic CPSS and 11 (22.4%) had an intrahepatic CPSS. Of the 49 dogs that had
267 surgery, 24 (49%) had complete attenuation and 25 (51%) had partial attenuation. The
268 25 dogs that had partial attenuation had repeat surgery a median of 110 days post-
269 operatively (range, 69-358 days). At the time of this repeat surgery, liver samples
270 from these 25 dogs were obtained for a second analysis, enabling comparison of
271 results with those from the first liver samples. At second surgery, 20 dogs tolerated
272 complete CPSS attenuation, three dogs had progressed to complete shunt occlusion
273 spontaneously (no flow on portovenography), and two dogs had developed multiple
274 acquired shunts. The liver of all dogs with CPSS at first and second surgery showed
275 characteristic changes consistent with portal hypoperfusion. No additional pathology
276 was noted. Liver samples were acquired from seven Beagle control dogs as controls.
277 The median age of control dogs was 628 days (range, 515-1544 days), which was

278 significantly older than for dogs with CPSS ($P = 0.036$). The liver of all control dogs
279 was histopathologically unremarkable. The results are summarised in Table 2.

280

281 Relative mRNA expression of IL-1 β ($P = 0.016$) and IL-6 ($P = 0.002$) were
282 both significantly greater for dogs with CPSS than for Beagle control dogs (Fig. 2).
283 Relative TLR4 mRNA expression was significantly greater in dogs with complete
284 attenuation compared with those with partial attenuation ($P = 0.011$; Fig. 2). Relative
285 TLR4 mRNA expression significantly increased following partial attenuation ($P =$
286 0.020 ; Fig. 2). Relative TLR4 mRNA expression was also compared between dogs
287 with poor portal blood flow and those with good portal blood flow on
288 portovenography (further details below). Relative TLR4 mRNA expression was
289 significantly greater in dogs with good portal blood flow compared to those with poor
290 portal blood flow on both pre-attenuation ($P = 0.004$) and post-attenuation
291 portovenograms ($P = 0.015$; Fig. 3, Table 3).

292

293 No significant associations were demonstrated for the relative mRNA
294 expression of TNF α or TLR2.

295

296 *Portovenogram grading*

297 Complete portovenograms were available for 47/49 dogs at first surgery and
298 21/25 dogs at second surgery. Pre-attenuation and post-attenuation portovenogram
299 grades at first surgery were significantly greater for dogs with complete attenuation
300 compared with dogs with partial attenuation ($P < 0.001$ for each). For dogs treated
301 with partial attenuation, there was a significant increase in both pre-temporary CPSS

302 attenuation portovenogram grade ($P < 0.001$) and post-temporary CPSS attenuation
303 portovenogram grade ($P = 0.001$) from first to second surgery (Table 4).

304

305 *Serum IL-6 concentration*

306 Serum samples taken before and at 24 and 48 h post-surgery from 22 dogs
307 with CPSS were analysed. The following breeds were included: Yorkshire terrier
308 ($n=4$), Norfolk terrier ($n=3$), West Highland white terrier ($n=3$), Jack Russell terrier
309 ($n=2$), Miniature Schnauzer ($n=2$), Shih Tzu ($n=2$), British bulldog ($n=1$), Crossbreed
310 ($n=1$), Golden retriever ($n=1$), Labrador ($n=1$), Miniature Dachshund ($n=1$), Shetland
311 sheepdog ($n=1$). The median age was 334.5 days (range, 114-2282 days). Nineteen
312 (86.4%) dogs had an extrahepatic CPSS and three (13.6%) had an intrahepatic CPSS.
313 Twelve dogs (54.5%) had complete attenuation and 10 (45.5%) had partial
314 attenuation.

315

316 Serum samples from seven healthy Beagles and five dogs undergoing
317 abdominal surgery were included as controls. The following breeds were included in
318 the abdominal surgery controls: Crossbreed ($n=2$), Golden retriever ($n=1$), Labrador
319 ($n=1$), Shar Pei ($n=1$). The dogs were undergoing abdominal surgery for the
320 investigation or treatment of insulinoma, adrenal carcinoma, splenic carcinoma,
321 phaeochromocytoma and extrahepatic biliary tract obstruction. The median age of
322 control dogs was 925 days (range, 526-4204 days); they were significantly older than
323 dogs with CPSS ($P < 0.001$).

324

325 There was no significant difference in pre-operative IL-6 concentrations
326 between dogs with CPSS (median, 0 pg/mL; range, 0-876.75 pg/mL) and control dogs

327 (median, 0 pg/mL; range, 0-40.66 pg/mL). The median IL-6 concentration at 24 h in
328 dogs with CPSS was 34.461 pg/mL (range, 0-483.726 pg/mL) and at 48 h was 8.137
329 pg/mL (range, 0-683.925 pg/mL; Fig. 4). In dogs with CPSS, there was a significant
330 difference in the concentration of IL-6 at the different time points ($P < 0.001$). Pair-
331 wise comparison of this data set confirmed that IL-6 at 24 h post-surgery was
332 significantly greater than pre-surgery ($P < 0.001$).

333

334 **Discussion**

335 We measured the LPS concentration in peripheral and portal blood samples
336 from dogs with CPSS and healthy Beagle control dogs. In normal animals and
337 humans, LPS from the gut is transported to the liver by the portal system and is
338 cleared by the Kupffer cells (Bradfield, 1974; Zlydaszyk and Moon, 1976; Jacob et
339 al., 1977). Therefore, LPS is routinely detected in the portal circulation, but only a
340 small amount is found in peripheral venous blood (Prytz et al., 1976; Jacob et al.,
341 1977). Portal LPS concentrations are significantly greater than peripheral
342 concentrations in both healthy humans and those with liver disease (Tachiyama et al.,
343 1988; Lumsden et al., 1988; Benten et al., 2011; Sanada et al., 2012). However, LPS
344 is increased in both the peripheral and portal blood of humans with liver cirrhosis due
345 to increased absorption from the gut and decreased hepatic clearance (Lumsden et al.,
346 1988; Tachiyama et al., 1988; Lin et al., 1995; Kaser et al., 2002). This increase is
347 thought to be due to either the shunting of blood past the liver via multiple acquired
348 shunts (MAS) or impaired LPS clearance due to liver pathology (Kaser et al., 2002).

349

350 Our study demonstrated that portal LPS concentration was significantly
351 greater than peripheral concentration in dogs with CPSS and Beagle control dogs.

352 Peripheral and portal LPS concentrations were also significantly greater in dogs with
353 CPSS compared to Beagle control dogs. These findings are consistent with those in
354 humans. It is unsurprising that LPS concentrations were significantly greater in dogs
355 with CPSS, as hepatic clearance is reduced as a consequence of shunting, as in
356 humans with MAS. However, in people with liver cirrhosis there is also increased
357 absorption of LPS from the gut, which is presumably not the case in dogs with CPSS.
358 Thus, the main reason for the increase in LPS is likely to be decreased hepatic
359 clearance. A previous study measured LPS in the peripheral and portal blood of 10
360 dogs with CPSS compared with five control dogs using an LAL assay (Peterson et al.,
361 1991). Contrary to our study, there were no significant differences in LPS
362 concentrations between peripheral and portal samples or between CPSS and control
363 dogs. The reason for this discrepancy could be due to differences in experimental
364 methodology or to variables in the dogs studied. Another study measured LPS
365 concentration in the portal vein, hepatic vein and caudal vena cava of 10 dogs with
366 experimentally created MAS and six control dogs using an LAL assay (Howe et al.,
367 1997). In agreement with our study, LPS concentration was significantly greater in all
368 vessels for dogs with MAS compared with control dogs.

369

370 In our study, and in the two canine studies mentioned above, an LAL assay
371 was used to measure plasma LPS concentration. The LAL assay has been commonly
372 used to measure plasma LPS in people with liver disease (Jacob et al., 1977; Lumsden
373 et al., 1988; Tachiyama et al., 1988; Lin et al., 1995; Benten et al., 2011). Many
374 variables can affect assay results and there is considerable variation in methodology
375 between studies, which can affect the sensitivity and reliability of the LAL assay
376 (Novitsky, 1994; Hurley, 1995). The endotoxin activity assay (EAA) is a more recent

377 technique for measuring endotoxin and uses chemiluminescence of neutrophil activity
378 (Romaschin et al., 1998; Marshall et al., 2002). It has been used in both humans and
379 dogs and is considered to be more accurate than the LAL method (Marshall et al.,
380 2002; Kjelgaard-Hansen et al., 2008; Sanada et al., 2011). It is possible that inclusion
381 of the EAA test in our methodology might have improved the reliability of our results,
382 but this test was not available to us for logistical and financial reasons.

383

384 Our findings demonstrated that LPS concentration was increased in the portal
385 blood of dogs with CPSS, most likely due to decreased hepatic clearance, suggesting
386 that reduced delivery of portal blood to the liver is accompanied by reduced LPS
387 delivery to the liver. This is consistent with the concept that portal delivery of LPS to
388 the liver is a potential trigger for the hepatic response to attenuation. Surgical CPSS
389 attenuation increases portal blood flow and hence increases LPS delivery to the liver.
390 Following CPSS attenuation, normalisation of LPS concentrations is expected due to
391 increased hepatic clearance. However, follow-up samples were not available so this
392 was not assessed.

393

394 We have demonstrated that there was a significant increase in serum IL-6 at
395 24 h following CPSS attenuation. IL-6 is a key mediator of the initial stages of
396 hepatic regeneration and rapid increases in hepatic and serum IL-6 are observed
397 following PH in rodents (Cressman et al., 1996; Sakamoto et al., 1999; Iwai et al.,
398 2001; Aldeguer et al., 2002). Increased portal blood flow results in stimulation of
399 Kupffer cells and release of IL-6, an initiator of regeneration (Decker, 1990; Meijer et
400 al., 2000; Abshagen et al., 2007; Riehle et al., 2008). The increase in IL-6 in dogs
401 with CPSS following attenuation could, at least in part, be due to increased hepatic

402 blood flow and IL-6 release as part of liver regeneration. However, there is another
403 possible explanation for the increase in IL-6. Abdominal surgery in humans is
404 associated with an inflammatory response, resulting in increased IL-6 in the
405 peripheral and portal blood (Cruickshank et al., 1990; Di Padova et al., 1991; Baigrie
406 et al., 1992; Glaser et al., 1995; Biffl et al., 1996; Kimura et al., 1998). Logically, the
407 increase in serum IL-6 following CPSS surgery might also be due to surgical trauma.
408 A control group of dogs undergoing laparotomy for reasons unrelated to CPSS
409 attenuation with pre- and post-operative serum samples would have provided more
410 information on the specificity of post-operative increase in IL-6.

411

412 Significant post-operative increases in serum IL-6 are seen in humans
413 undergoing PH for tumour resection and in individuals donating or receiving liver
414 transplants (Kimura et al., 1998, 1999; Hu et al., 1999; Asakura et al., 2000; Chijiwa
415 et al., 2002; Slotwinski et al., 2002). One study demonstrated a significant increase in
416 serum IL-6 at 24, 72 and 168 h post-hepatectomy for liver donation, but there was no
417 significant increase post-hepatectomy for benign neoplasia (Slotwinski et al., 2002).
418 The study concluded that in humans with normal livers, IL-6 increased following
419 partial hepatectomy, consistent with hepatic regeneration. This is similar to the
420 findings of our study, although IL-6 rapidly returned to normal, whereas it remained
421 increased in people following hepatectomy. In other studies of abdominal surgery in
422 people, IL-6 levels increase rapidly to peak at 4-48 h post-operatively, but can remain
423 increased for 48-72 h (Di Padova et al., 1991; Baigrie et al., 1992; Biffl et al., 1996).
424 The reason for these discrepancies is unclear, but could be related to differences in the
425 nature of the surgery and therefore the hepatic response, species differences,
426 differences in methodology and assay sensitivity. However, pre-existing liver disease

427 is associated with increased IL-6 and this could affect post-operative changes (Hu et
428 al., 1999; Slotwinski et al., 2002). Similarly, dogs with liver disease have increased
429 serum IL-6 concentrations compared with healthy dogs (Neumann et al., 2012). In a
430 recent study, plasma IL-6 concentrations were increased in dogs with CPSS
431 (Kilpatrick et al., 2014). Our study did not find a significant difference in serum IL-6
432 concentrations between CPSS and control dogs. However, it is possible that
433 differences in the populations and methodology could be responsible for this
434 discrepancy. Additionally, our study had a relatively small number of control dogs,
435 perhaps resulting in a type II statistical error.

436

437 We measured the expression of IL-1 β , IL-6 and TNF α mRNA in liver tissue.
438 The expression of both IL-1 β and IL-6 mRNA in liver tissue were significantly
439 greater in dogs with CPSS compared to Beagle control dogs. IL-1 β , IL-6 and TNF α
440 are inflammatory cytokines that are released by Kupffer cells in response to
441 stimulation by LPS (Shirahama et al., 1988; Decker et al., 1989; Busam et al., 1990).
442 IL-6 and TNF α are initiators of regeneration and IL-1 β inhibits regeneration (Boulton
443 et al., 1997; Fausto et al., 2006b; Riehle et al., 2011). Therefore, it seems incongruous
444 that both IL-6 and TNF α are increased in an underdeveloped CPSS liver. As
445 mentioned above, abdominal surgery initiates an inflammatory response accompanied
446 by increases in serum IL-6 and IL-1 β ; increases in IL-1 β precede those for IL-6
447 (Baigrie et al., 1992; Glaser et al., 1995; Kimura et al., 1998). It is possible that liver
448 biopsy would result in similar increases in IL-1 β and IL-6 in the traumatised tissue.
449 Therefore, the increased cytokine expression in CPSS liver tissue could be due to
450 acute release following surgical trauma. As control liver tissue was obtained post-
451 mortem, there might not have been similar increases in cytokine expression.

452 However, this potential explanation is conjecture and remains unproven. Several
453 studies have shown that dogs with CPSS, and in particular those with hepatic
454 encephalopathy, have evidence of generalised inflammation with increased serum IL-
455 6, plasma C-reactive protein (CRP) and systemic inflammatory response syndrome
456 scores (Gow et al., 2012; Kilpatrick et al., 2014; Tivers et al., 2014c, 2015). It is
457 possible that more generalised increases in IL-1 β and IL-6 as a result of pre-existing
458 inflammation could be responsible for the increased hepatic expression of these
459 cytokines. It is also possible that differences in IL-1 β and IL-6 between the CPSS and
460 control dogs could have been due to differences in breed and age between the two
461 groups. However, if this were the case, a physiological reason for this difference is
462 unclear.

463

464 We measured the hepatic mRNA expression of TLR2 and TLR4. TLR4
465 mRNA expression was significantly increased in dogs with CPSS tolerating complete
466 attenuation compared to those which tolerated only partial attenuation. Dogs with
467 well-developed intrahepatic portal vasculature on portovenography had significantly
468 increased TLR4 mRNA expression. Additionally, TLR4 mRNA expression
469 significantly increased following partial attenuation. In contrast, no significant
470 differences were identified for TLR2. This finding might be because TLR2 plays a
471 major role in detection of Gram-positive bacteria, recognising components of the cell
472 wall including peptidoglycan, lipoteichoic acid and lipoproteins (Yoshimura et al.,
473 1999). Gram-positive bacteria are not the predominant component of typical gut
474 flora. The absence of a significant difference in TLR2 mRNA expression in
475 conjunction with a significant increase in TLR4 mRNA expression increases the

476 likelihood that these data reflect a specific interaction between gut flora pathogen-
477 associated molecular patterns and the hepatic response to CPSS attenuation.

478

479 TLR4 is expressed by Kupffer cells and binds LPS, enabling circulatory
480 clearance of LPS (Freudenberg et al., 1982; Fenton and Golenbock, 1998). LPS is
481 very important for normal hepatic regeneration (Cornell, 1985a, b, 1990; Gao et al.,
482 1999). Kupffer cell release of IL-6 and TNF α in response to LPS is implicated in the
483 initiation of hepatic regeneration (Fausto, 2006a). Increased expression of TLR4
484 mRNA suggests increased LPS binding capacity in dogs with CPSS and good portal
485 blood flow and in those able to tolerate complete attenuation. As partial attenuation
486 increases portal blood flow, increased TLR4 mRNA is therefore consistent with
487 increased LPS delivery. This provides further evidence that TLR4 and blood flow are
488 important in the hepatic response to surgery. These findings demonstrate that TLR4
489 mRNA expression is linked with portal blood flow and in the response to surgical
490 attenuation in dogs with CPSS. We have previously shown that the hepatic expression
491 of hepatocyte growth factor (HGF) and methionine adenosyltransferase 2 A, which
492 are both markers of hepatocyte replication, are significantly increased following
493 partial CPSS attenuation (Tivers et al., 2014a). We have also shown that vascular
494 endothelial growth factor receptor 2 (VEGFR2) is significantly associated with the
495 degree of portal blood flow and significantly increases following partial CPSS
496 attenuation (Tivers et al., 2014b). In addition, these studies also demonstrated that
497 there were significant increases in HGF and VEGF immediately following CPSS
498 surgery (Tivers et al., 2014a, b). These data suggest that both hepatic regeneration, in
499 the form of hepatocyte replication and angiogenesis, are associated with CPSS
500 attenuation. The findings of the current study are in broad agreement with these

501 findings and support the concept that activation of Kupffer cells via TLR4 binding of
502 LPS could be involved in this process. Further work is needed to explore this concept.

503

504 There are a number of limitations to the current study that must be taken into
505 account. The number of dogs included in the study was relatively small, particularly
506 for the measurement of plasma LPS and in the control groups for the other
507 experiments. Larger group size might have allowed further statistically significant
508 findings to be identified. Nevertheless, we were able to identify a number of findings
509 that were both biologically relevant and statistically significant. In addition, the
510 experimental Beagles used as control dogs were significantly older than the dogs with
511 CPSS. Consequently, it is possible that the differences detected in LPS concentration
512 and cytokine mRNA expression could have been related to breed or age rather than to
513 CPSS.

514

515 **Conclusions**

516 Our results have demonstrated that portal LPS is increased in dogs with CPSS,
517 consistent with decreased hepatic clearance due to shunting. In addition, hepatic
518 TLR4 mRNA expression was significantly associated with portal blood flow and
519 significantly increased following CPSS attenuation. This suggests that LPS binding
520 capacity via TLR4 is linked to blood flow and the degree of portal development. This
521 provides supporting evidence for the concept that LPS triggers liver regeneration via
522 Kupffer cell binding and signalling following CPSS attenuation. Further
523 investigations are warranted to explore this mechanism in more depth.

524

525 **Conflict of interest statement**

526 None of the authors of this paper has a financial or personal relationship with
527 other people or organisations that could inappropriately influence or bias the content
528 of the paper.

529

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538

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856 **Table 1**

857 Table showing details of reference gene and gene of interest primer pairs for quantitative PCR.

858

Gene	Primer sequences	PCR amplicon length (bp)	Genbank accession number	Primer sequence reference
HMBS	Forward: TCACCATCGGAGCCATCT Reverse: GTTCCCACCACGCTCTTCT	112	XM546491	Peters et al., 2007
RPL13A	Forward: GCCGGAAGGTTGTAGTCGT Reverse: GGAGGAAGGCCAGGTAATTC	87	AJ388525	Peters et al., 2007
RPL32	Forward: TGGTTACAGGAGCAACAAGAAA Reverse: GCACATCAGCAGCACTTCA	100	XM_848016	Peters et al., 2007
RPS18	Forward: TGCTCATGTGGTATTGAGGAA Reverse: TCTTATACTGGCGTGGATTCTG	116	XM_532106	Peters et al., 2007
IL-1 β	Forward: TCTCCCACCAGCTCTGTAACAA Reverse: GCAGGGCTTCTTCAGCTTCTC	80	Z70047	Wang et al., 2007
IL-6	Forward: TCCTGGTGATGGCTACTGCTT Reverse: GACTATTTGAAGTGGCATCATCCTT	78	U12234	Wang et al., 2007
TNF α	Forward: GAGCCGACGTGCCAATG Reverse: CAACCCATCTGACGGCACTA	79	Z70046	Wang et al., 2007
TLR2	Forward: AGTGGCCAGAAAAGCTGAAA Reverse: ATCCAGTTGCTCCTTCGAGA	263	NM001005264	House et al., 2008
TLR4	Forward: CAAAATCCCCAACAACATCC Reverse: TGGTTTAGGCCCTGATATGC	171	NM001002950	House et al., 2008

859 bp, base pairs; HMBS, hydroxymethyl-bilane synthase; RP, ribosomal protein; TNF, tumour necrosis factor; TLR, toll-like receptor

860 **Table 2**

861 Relative mRNA expression of cytokines and toll-like receptors (normalised with respect to four liver specific reference genes) in liver biopsies
 862 from 49 dogs with congenital portosystemic shunts (CPSS) and seven Beagle control dogs. Results are presented as median and range.

Gene	Control compared to CPSS			Complete attenuation compared to partial attenuation			Before and after partial attenuation (n=25)		
	Control ^a (n=7)	CPSS (n=49)	<i>P</i>	Partial (n=25)	Complete (n=24)	<i>P</i>	Before partial attenuation	After partial attenuation	<i>P</i>
IL-1 β	3.351 (2.361-5.723)	11.172 (1.654-919.494)	0.016 ^b	7.817 (1.654-197.141)	11.849 (2.826-919.494)	0.052	7.817 (1.654-197.141)	8.981 (1.975-169.281)	0.800
IL-6	2.569 (1.288-3.463)	9.473 (1.581-332.589)	0.002 ^b	8.310 (1.914-332.589)	13.044 (1.581-229.601)	0.421	8.310 (1.914-332.589)	5.543 (1.548-207.752)	0.155
TNF α	2.554 (2.158-4.402)	2.818 (1.509-11.364)	0.772	2.736 (1.509-5.917)	2.833 (1.544-11.364)	0.601	2.736 (1.509-5.917)	2.748 (1.326-14.032)	0.174
TLR2	2.041 (1.700-3.525)	2.241 (1.396-9.904)	0.298	2.204 (1.396-5.046)	2.242 (1.602-9.904)	0.401	2.204 (1.396-5.046)	2.372 (1.189-12.289)	0.148
TLR4	4.340 (4.005-7.049)	5.067 (1.581-20.575)	0.396	4.337 (1.581-14.505)	6.189 (3.183-20.575)	0.011 ^b	4.337 (1.581-14.505)	6.065 (1.627-11.617)	0.020 ^b

863 IL, interleukin; TNF, tumour necrosis factor; TLR, toll-like receptor

864 ^a Beagle dogs865 ^b Statistically significant value ($P \leq 0.05$)

866

867 **Table 3**

868 Relative mRNA expression of toll-like receptor 4 (TLR4), normalised with respect to four liver specific reference genes, in liver biopsies from
 869 47 dogs with congenital portosystemic shunts as related to portal blood flow on pre-attenuation and post-attenuation portovenogram, at first
 870 surgery. Results are presented as median and range.

Gene	Pre-attenuation portal blood flow			Post-attenuation portal blood flow		
	Poor (35 dogs)	Good (12 dogs)	<i>P</i>	Poor (12 dogs)	Good (35 dogs)	<i>P</i>
TLR4	4.607 (1.581-14.505)	7.638 (3.423-20.575)	0.004 ^a	4.271 (1.581-8.060)	5.513 (2.188-20.575)	0.01 ^a

871 ^a Statistically significant value ($P \leq 0.05$)

872

873 **Table 4**

874 Portovenogram grade before and after temporary congenital portosystemic shunt
 875 attenuation in 21 dogs at first and second surgery. This group of dogs all had a partial
 876 attenuation at the first surgery. There was a significant increase in portovenogram
 877 grade for both pre-attenuation and post-attenuation from first to second surgery ($P <$
 878 0.001 and $P = 0.001$, respectively).

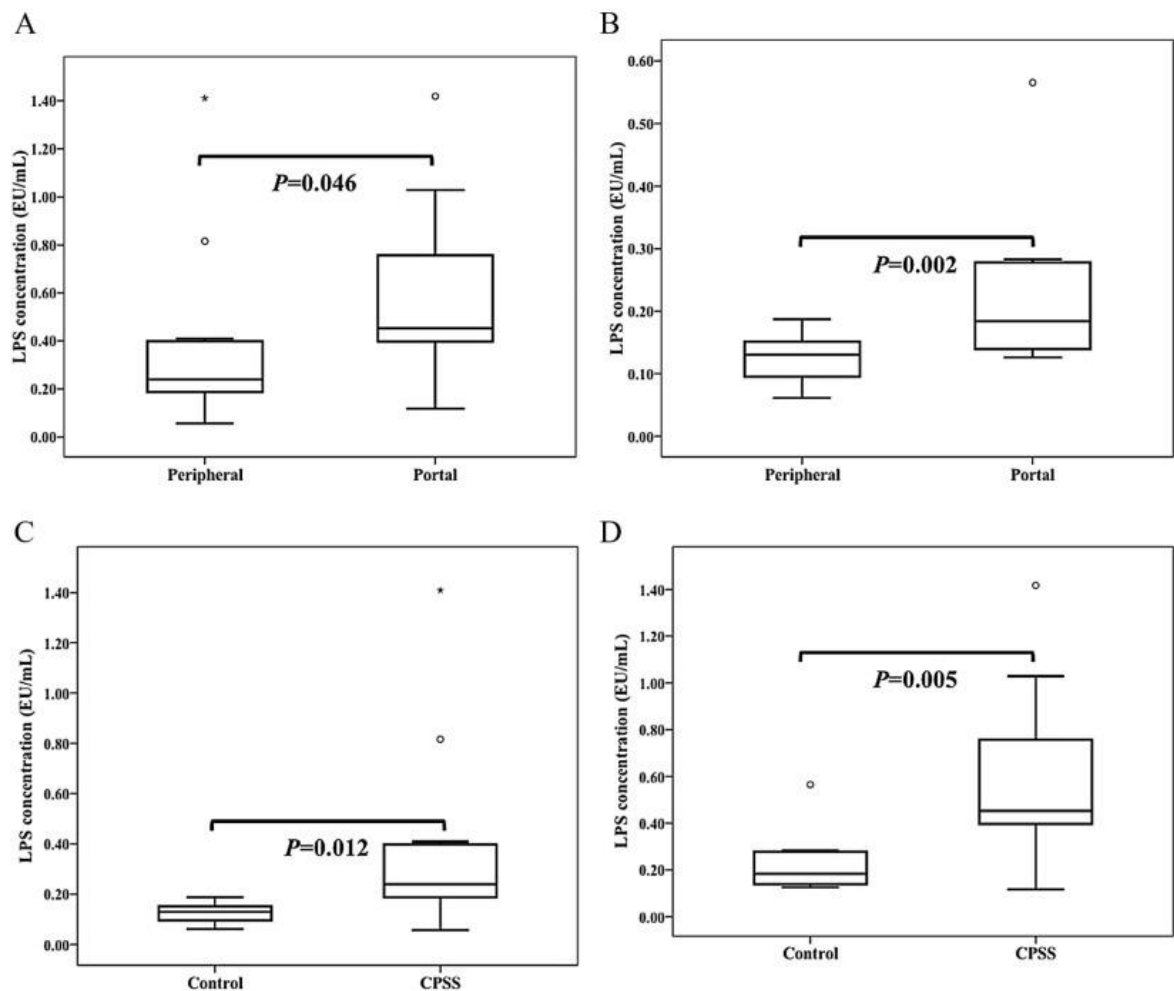
879

Timing of assessment	<i>n</i> (%) dogs for each portovenogram grade			
	Grade 1	Grade 2	Grade 3	Grade 4
1 st Surgery Pre-attenuation	18 (85.7)	3 (14.3)	0 (0)	0 (0)
1 st Surgery Post-attenuation	0 (0)	12 (57.1)	9 (42.9)	0 (0)
2 nd Surgery Pre-attenuation	2 (9.5)	8 (38.1)	6 (28.6)	5 (23.8)
2 nd Surgery Post-attenuation	0 (0)	4 (19.0)	6 (28.6)	10 (52.4)

880

881 **Figure legends**

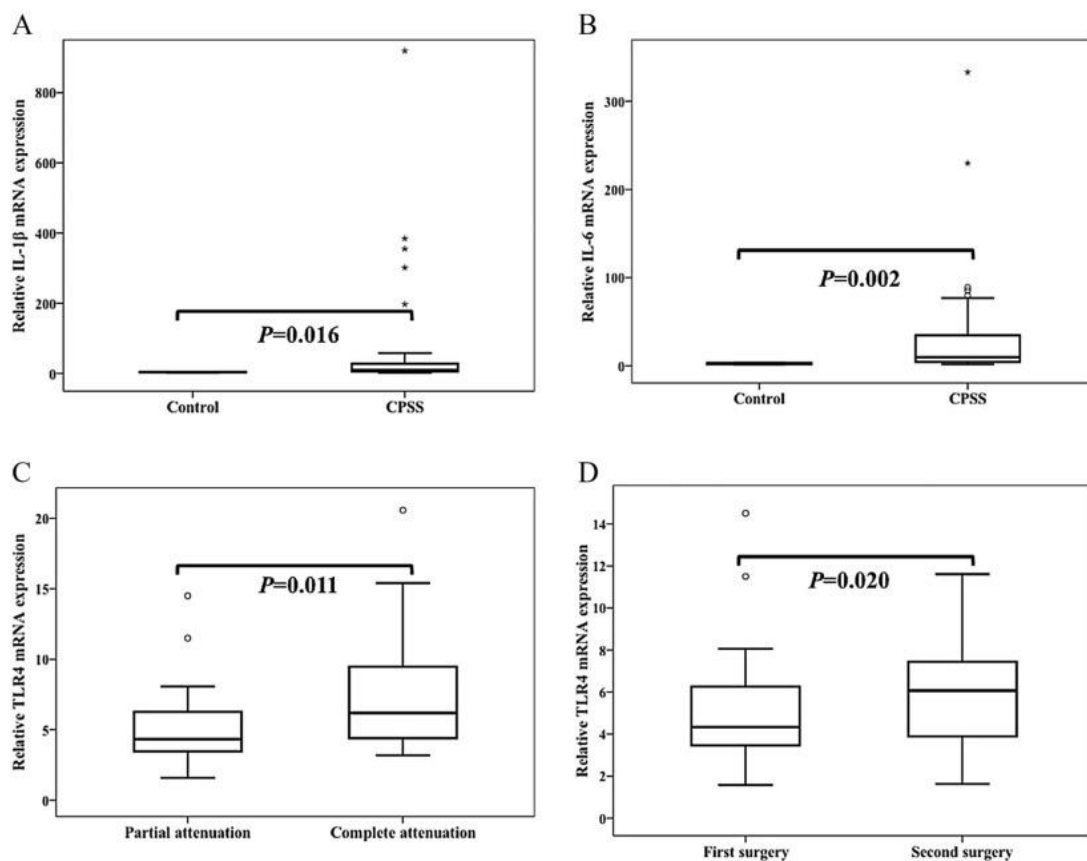
882 Fig. 1. Measurement of lipopolysaccharide (LPS) concentration (endotoxin units
883 [EU]/mL) in peripheral and portal plasma from 13 dogs with congenital portosystemic
884 shunts (CPSS) and nine healthy Beagle control dogs using a limulus ameocyte lysate
885 (LAL) assay. Statistical significance is highlighted with the corresponding *P* value.
886 (A) Peripheral and portal LPS concentration in dogs with CPSS. (B) Peripheral and
887 portal LPS concentrations in Beagle control dogs. (C) Peripheral LPS concentration in
888 Beagle control dogs compared with dogs with CPSS. (D) Portal LPS concentration in
889 Beagle control dogs compared with dogs with CPSS.



890

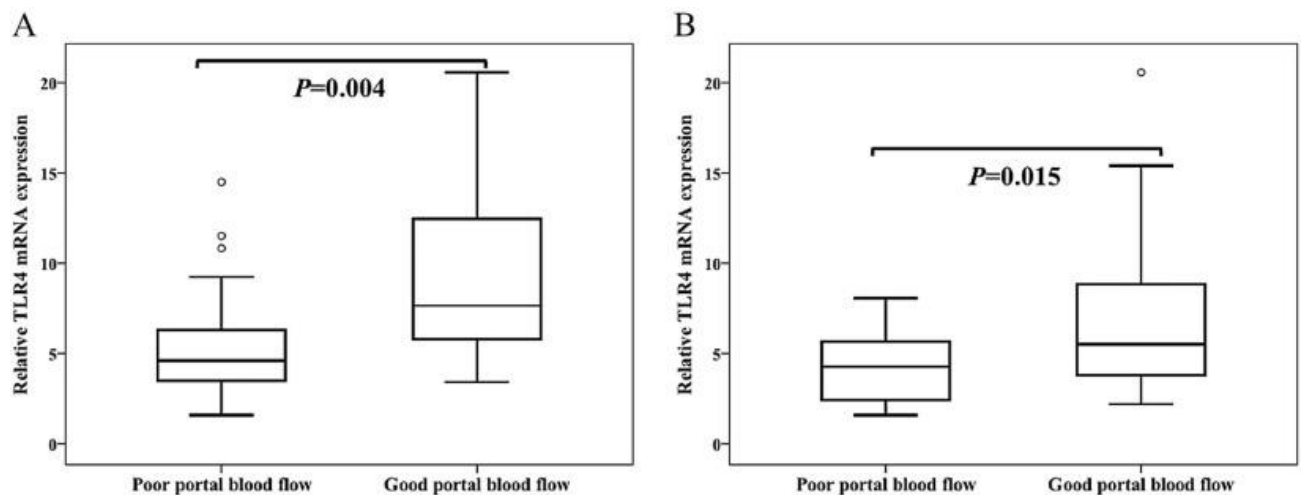
891

892 Fig. 2. Relative cytokine and toll-like receptor mRNA expression (normalised with
 893 respect to four liver specific reference genes) in liver biopsies from 49 dogs with
 894 congenital portosystemic shunts (CPSS) and seven Beagle control dogs. The graphs
 895 show statistically significant findings for the five genes assessed. Statistical
 896 significance is highlighted with the corresponding *P* value. (A) Interleukin 1 beta (IL-
 897 1 β) mRNA expression in Beagle control dogs compared with dogs with CPSS. (B)
 898 Interleukin 6 (IL-6) mRNA expression in Beagle control dogs compared with dogs
 899 with CPSS. (C) Toll-like receptor 4 (TLR4) mRNA expression in dogs with CPSS
 900 tolerating a partial attenuation compared with those tolerating a complete attenuation.
 901 (D) TLR4 mRNA expression in dogs with CPSS at first surgery compared with
 902 second surgery.



903
 904

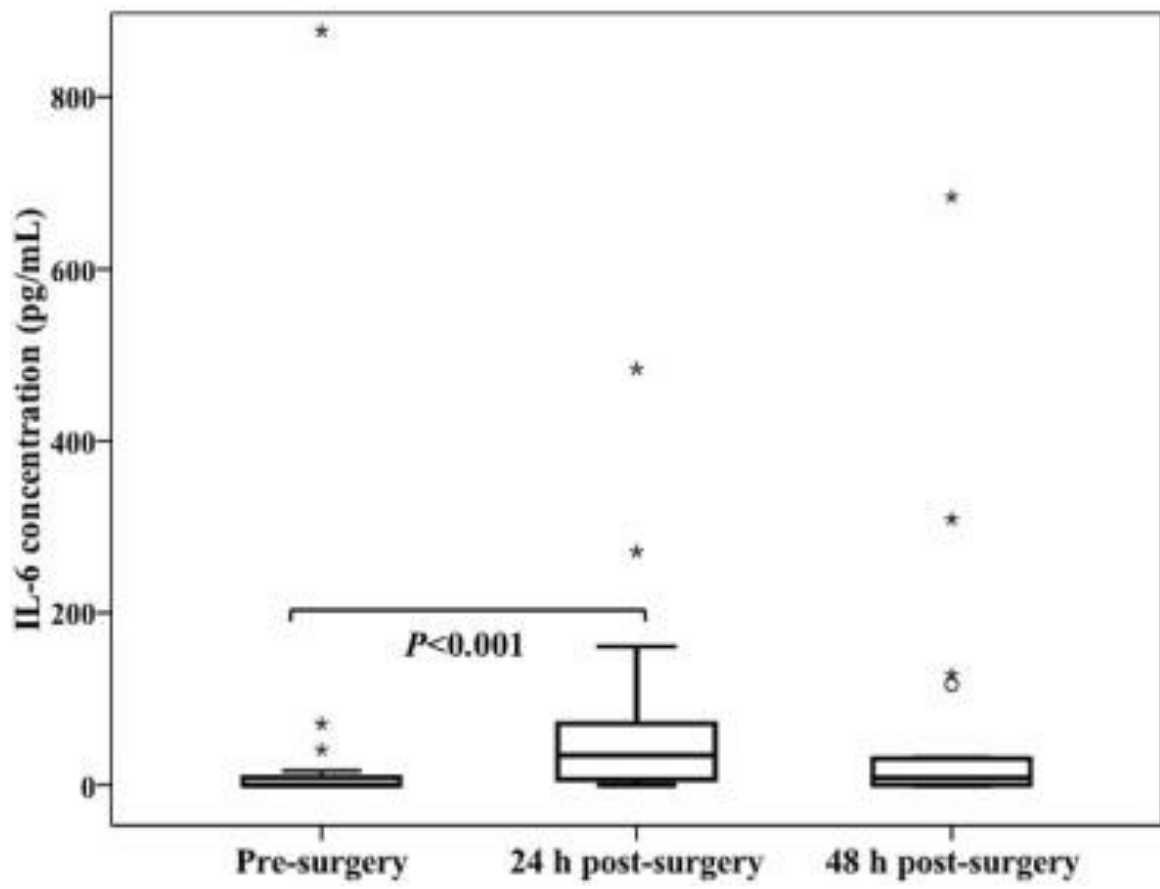
905 Fig. 3. Relative mRNA expression of toll-like receptor 4 (TLR4), normalised with
906 respect to four liver specific reference genes, in liver biopsies from 47 dogs with
907 congenital portosystemic shunts (CPSS) as related to portal blood flow on pre-
908 attenuation and post-attenuation portovenogram. Portovenogram grades of 1 and 2
909 were considered poor portal blood flow and portovenogram grades of 3 and 4 were
910 considered good portal blood flow. Statistical significance is highlighted with the
911 corresponding *P* value. (A) TLR4 mRNA expression in dogs with CPSS with poor
912 portal blood flow compared with dogs with CPSS with good portal blood flow on pre-
913 attenuation portovenogram. (B) TLR4 mRNA expression in dogs with CPSS with
914 poor portal blood flow compared with dogs with CPSS with good portal blood flow
915 on post-attenuation portovenogram.



916

917

918 Fig. 4. Serum interleukin 6 (IL-6) concentration in 22 dogs with congenital
919 portosystemic shunts pre-surgery and at 24 and 48 h post-surgery. IL-6 concentration
920 was measured using a canine IL-6 ELISA kit. There was a significant difference in
921 the concentration of IL-6 at the different time points ($P < 0.001$). Pair-wise
922 comparison of this data set confirmed that IL-6 at 24 h post-surgery was significantly
923 greater than pre-surgery ($P < 0.001$).



924